

REMARKS

Priority Document

Applicants supplied a certified copy of the foreign priority application to the International Bureau to meet the requirements for filing a PCT International Application. As such the International Bureau should have forwarded a photocopy of the certified priority document to the United States Patent and Trademark Office as a Designated Office. The priority document sent to the U.S. Office of PCT Operations from the International Bureau is acceptable to establish that applicants have filed a certified copy of the priority document.

Specification

Applicants have provide herewith an amended specification to meet the guidelines requested by the Examiner. The term "naked" has been replaced with the term "--nude--." Proper headings have been included in Table 1. The description of Figure 2A and B has been amended to replace the term "gray" with -- black --. Also the term "sensibilization" as used through the application has been replaced with -- sensitization--. Applicants have made the required amendments to the specification and request that all objections to the specification be withdrawn.

Rejection of Claims and Traversal Thereof

In the January 17, 2002 Office Action,

claims 1-9 were rejected under 35 U.S.C. §112, first paragraph;

claims 1-6 were rejected under 35 U.S.C. §112, second paragraph; and

claims 1-6 were rejected under 35 U.S.C. §101.

These rejections are hereby traversed and reconsideration of the patentability of the pending claims is therefore requested in light of the following remarks.

Rejection under 35 U.S.C. § 112, first paragraph

In the January 17, 2002 Office Action, claims 1-9 were rejected under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains or with which it is most nearly connected, to make and/or use the invention.

According to the Office the disclosure fails to provide adequate guidance in a number of areas. As such, applicants will address each of the Office's remarks and contentions individually.

1. The Office contends that "Applicants have not provided guidance in the specification toward specific treatment protocols or a pharmaceutical composition containing a chemotherapeutic agent and AAV-2 that would show AAV-2 infection is capable to reduced radiotherapy or chemotherapy-induced tumor resistance in patients." In response, applicants argue that ample disclosure has been provided in the instant application for practicing the present invention. For instance, on page 4, line 18 to page 5, line 22 of the amended specification submitted herewith, there is a complete discussion on methods for administration of the AAV-2 virus. The AAV-2 virus can be administered in a physiological common salt solution, Ringer's solution or PBS solution. The effective amount of the virus dose employed is defined to include 10^9 - 10^{10} AAV-2 particle/kg body weight. Clearly, one skilled in the art would recognize that the amount of the chemotherapeutic agent can be determined by a physician and is dependent on the patient's sex, weight, severity of the disease, kind of administration and planned duration of administration. A pharmaceutical composition including both chemotherapeutic agent and AAV-2 is described specifically in lines 8-14 on page 5. Thus, one skilled in the art without the exercise of inventive skill or undue experimentation could determine an effective dose, especially because the disclosed chemotherapeutic agents are well known in the art and have been used for many years to treat cancers.

2. According to the Office,

"The specification fails to disclose a working example for a method to treat cancer patients with a combination of a chemotherapeutic agent and an AAV-2. The specification also fails to disclose a composition comprising such a combination with therapeutic effect in cancer patients."

Applicants vigorously disagrees. The Office has faulted the applicants for not showing efficacy of the claimed invention in humans. Applicants submit that the efficacy of the compositions of this invention is fully and rigorously established by applicants' empirical determinations as set forth in Examples 1-3. Applicants not only provided *in vitro* data in Examples 1 and 2 but also showed that the *in vitro* data was predictive of *in vivo* utility and efficacy in Example 3. Example 3 provides test results for the efficacy of the claimed composition in a murine model (nude mouse) which is considered an acceptable animal testing model because the nude mouse is known to exhibit results that can be extrapolated to human pathology and efficacy. Test data resulting from tests performed on the nude mouse model provide actual evidence that applicants' claimed invention enhanced sensitivity of small cell lung cancer cells (SCLC) and reversed chemotherapy-resistance when chemotherapeutic agent were combined with AAV-2 infection. As shown in Figure 3, treatment with the chemotherapeutics resulted in a rapid decrease in tumor volumes and complete regression after 3 weeks of treatment. The combination of chemotherapy with AAV-2 infection led to a more pronounced decline of tumor volume compared with animals that received only chemotherapy, indicating a sensitization of drug-treated tumor cells. Treatment was interrupted after regression of the tumors and was resumed when relapses occurred. Treatment of recurrences was less efficient in animals that received only drug treatment but when treated with AAV-2, the resistance to treatment was completely reversed. Tumors treated with the combination had completely regressed in comparison to the tumors treated with just the chemotherapeutic drug.

Applicants' specification contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented. Thus applicants' specification must be taken as in compliance with the enabling requirement of the first paragraph of Section 112 unless there is reason to doubt the objective truth of the statements contained therein. *In re Marzocchi*, 169 USPQ 367 (CCPA 1971). The Office has not put forth any reasoning or evidence to show that the statements made in the specification are not true and that the claimed invention would not be effective in a human patient.

Applicants remind the Office that the standard for enablement and thus patentability is not the same as that required for drug marketing approval by the Federal Drug Administration. See *Scott v. Finney*, 32 USPQ2d 115 (Fed. Cir. 1994). Notably, the Court in *In re Jolles*, 206 USPQ 885 (CCPA 1980) ruled that a correlation between animal models and clinical efficacy does not mean that clinical studies must be carried out. Thus, patentability and enablement do not hinge on the outcome of human clinical trials and *in vivo* testing results are considered predictive of efficacy in humans.

The National Institute of Health (NIH) and other cancer researchers recognize the nu/nu mouse model as a standard screening model for determining effectiveness of new chemotherapeutic compounds. The nude mouse has played a key role in many medical research advances. Applicants have included in Appendix C several articles discussing the use of mice for medical research. As stated in Reference 1, the nude mouse was instrumental in development of vaccines for whooping cough, yellow fever and contributes to research on cancer. Reference 2 discusses the use of mice in cancer and this allows the study of human cancer cells without risking human life.

Applicants include herewith in Appendix D several articles that demonstrates the effectiveness of the mouse as an indicator of efficacy of cancer treatment in humans. For instance, in Reference 1, it was shown that the compound C242-DM1, a chemotherapeutic agent, eliminated human colorectal tumors in SCID (similar to nude mouse) mice at very low doses. In addition, the compound was administered to humans with colorectal cancers and found consistent decreases in colon cancer marker CEA in several patients.

Reference 2, discusses the use of AG3340 as an effective chemotherapeutic agent that showed marked improvement in treatment of humans and mice. The administration of the compound to mice resulted in a decrease in tumor growth of small cell lung cancer tumors by up to 65% as compared to controls. Likewise, the efficacy of the compound found effective in mice was found effective in humans and reduced tumors sizes or at least stabilized the tumors.

Reference 3, describes the efficacy of Virulizin, an immunotherapeutic agent that was effective in mouse models of human pancreatic cancer and also found effective in humans suffering from cancer. Thus, it is well known in the art that a mouse is an effective and acceptable animal model wherein effective results in animal testing can be extrapolated to human efficacy.

As stated above, FDA approval is not a prerequisite for finding a compound useful within the meaning of patent law. This very issue was addressed in *In re Brana*, 34 USPQ2d 1436 (Fed. Cir. 1995). The Federal Circuit reversed a final rejection under 35 U.S.C. §112, first paragraph for claimed compounds that were tested in a murine model and found effective as an antitumor compound, but rejected by the Examiner. The *Brana* Court further ruled that the murine model tests were sufficient to convince one of ordinary skill in the art that the claimed compounds were useful as antitumor agents. The Court specifically stated that:

“Were we to require Phase II testing in order to prove utility, the associated cost would prevent many companies from obtaining patent protection on promising new invention, thereby eliminating an incentive to pursue, through research and development, potential cures in many crucial areas, such as the treatment of cancer. In view of all the foregoing, we conclude that applicants’ disclosure complies with the requirements of 35 U.S.C. section 112, Para.1.”

Even without Phase II testing, one skilled in the art would recognize that the effectiveness of the presently claimed invention in the acceptable *in vivo* murine model for cancer treatment would also show human efficacy.

The claims as now amended recite applicants’ invention in terms fully supported in the disclosure defining the subject matter sought to be patented. The claims thus are in compliance with the enablement requirement of the first paragraph of section 112. Applicants correspondingly respectfully request the withdrawal of the rejection of claims 1-9 under §112, first paragraph.

Rejection under 35 U.S.C. § 112, second paragraph

Claims 1-6 were rejected under 35 U.S.C. §112, second paragraph because according to the Office “[t]he claim does not set forth any steps involved in the method/process.” Applicants have amended independent claim 1 and therefore all claims depending therefrom to recite the claimed invention in acceptable method steps. As such, the amendment of claims 1-6 obviates the rejection under §112, and as such applicants request the withdrawal of this rejection.

Rejection under 35 U.S.C. § 101

Claims 1-6 were rejected under 35 U.S.C. §101 because the claims did not set forth any steps involved in a process and therefore not proper process claims under 35 U.S.C. §101. Applicants have amended independent claim 1 and therefore all claims depending therefrom by reciting acceptable method step terminology. As such, the amendment of claims 1-6 obviates the rejection under §101 and as such applicants request the withdrawal of this rejection.

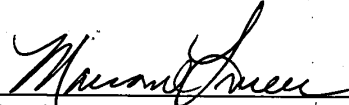
Fees Payable

Three new claims have been added beyond the number for which a fee has previously been paid, resulting in an added claims fee of \$69.00. A check in the amount of \$69.00 is submitted herewith in payment of the fee for the additional claims. The U.S. Patent and Trademark Office is hereby authorized to charge any additional amount necessary to the entry of this amendment, and to credit any excess payment, to Deposit Account No. 08-3284 of Intellectual Property/Technology Law.

CONCLUSION

Pending claims 1-12 meet all requirements of patentability and are in condition for allowance. If any issues remain outstanding, incident to allowance of the application, the Examiner is requested to contact the undersigned attorney at (919) 419-9350 to discuss their resolution, in order that this application may be passed to issue at an early date.

Respectfully submitted,



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APPENDIX A

Version with Markings to Show Changes Made

In the Claims:

- 1) A method for lowering the [Use of adeno-associated viruses for lowering the radiotherapy-induced or] chemotherapy-induced resistance in a patient[s] being treated with a chemotherapeutic agent for [suffering from] a cancer, the method [which is to be treated by radiotherapy or chemotherapy] comprising: infecting the patient with an effective amount of AAV-2 to lower the chemotherapy-induced resistance to the chemotherapeutic agent, in combination with administering a chemotherapeutic agent .
- 2) The method [Use] according to claim 1, wherein the [adeno-associated viruses] AAV-2 is [are] used in a dose of 10^9 - 10^{10} AAV particles/kg body weight.
- 3) The method [Use] according to claim 1 [any of claims 1 to 2], wherein the chemotherapeutic agent is selected from the group consisting of: cisplatin, etoposide and cisplatin/etoposide.
- 4) The method [Use] according to any of claims 1 to 3, wherein the cancer to be treated by [radiotherapy or] chemotherapy is a colon cancer, pancreatic carcinoma or brain tumor or small cell lung carcinoma.
- 5) The method [Use] according to any of claims 1 to 4, wherein the use is made intravenously, cutaneously, orally or intratumorally.
- 6) The method [Use] according to any of claims 1 to 5, wherein the infecting with the AAV-2 [use] is made before, after or simultaneously with a chemotherapy or radiotherapy.

- 7) A pharmaceutical composition containing a chemotherapeutic agent and an effective dose of AAV-2 to reverse chemotherapy-induced resistance in patients suffering from small cell lung carcinoma.
- 8) The pharmaceutical composition according to claim 7, wherein the chemotherapeutic agent is [cisplatin and/or etoposide] selected from the group consisting of: cisplatin, etoposide and cisplatin/etoposide.
- 9) The pharmaceutical composition according to claim 7 or 8, wherein the composition is formulated in a member selected from the group consisting of: [it is in] an injection solution, [or] infusion solution, an aerosol spray or an ointment.

USE OF ADENO-ASSOCIATED VIRUSES FOR DECREASING THE
RADIOTHERAPY-INDUCED OR CHEMOTHERAPY-INDUCED RESISTANCE IN
CANCER PATIENTS

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CROSS-REFERENCE TO RELATED APPLICATIONS

This application is filed under the provisions of 35 USC §371 and claims the priority of
International Patent Application No. PCT/DE99/01711 filed June 8, 1999, which in turn
10 claims priority of German Patent Application No. 198 25 620.5 filed June 8, 1998.

BACKGROUND OF THE INVENTION

Field of Invention

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The invention relates to the use of adeno-associated viruses for decreasing the
radiotherapy-induced or chemotherapy-induced resistance in patients who suffer from a
cancer which is to be treated by radiotherapy or chemotherapy.

20 Description of Related Art

Along with the removal by surgery malignant diseases have been treated by radiotherapy
and/or chemotherapy thus far. However, the occurrence of side-effects, in particular the
development of resistances, limits the use of cytostatic agents. Because of these side-
25 effects chemotherapeutic agents can only be used to a limited extent. Thus, the dosage of a

chemotherapeutic agent can only be a dosage which the patient tolerates. However, in most cases such a dosage only achieves a minor curative effect.

SUMMARY OF THE INVENTION

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Therefore, it is the object of the present invention to reduce or attenuate the problem of resistance induced by radiotherapy or chemotherapy so as to improve the survival rate of cancer patients after a radiotherapy or chemotherapy. In addition, it shall be possible to reduce the subsequent doses of a radiotherapy or chemotherapy. The responsiveness of
10 tumor cells to radiotherapy or chemotherapy shall also be improved

This object is achieved by the subject matters defined in the claims.

According to the invention the development of a radiotherapy-induced or chemotherapy-
15 induced resistance in patients suffering from a cancer to be treated by radiotherapy or chemotherapy shall be reduced by using adeno-associated viruses.

It was found surprisingly that following a treatment with human non-pathogenic adeno-associated viruses (AAV) the tumor cells respond in a better way to a subsequent
20 chemotherapeutic or radiotherapeutic measure.

The AAV virus is a human parvovirus which requires co-infection with a helper virus for a

productive infection to take place. AAV infects human in their infancy and is considered non-pathogenic, since no human disease could be correlated with the AAV infection (Adv. in Vir. Res., 1987, 32, 43-306).

- 5 According to the inventors' insight, an AAV infection in combination with chemotherapeutic or radiotherapeutic measures increases the efficiency of a conventional therapy and reduces resistances which occur, so that a further therapy can be carried out in a more promising way than possible thus far. Any AAV viruses can be used according to the invention. The AAV-2 virus is used preferably.

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In animal experiments carried out with immunodeficient [naked] Nude mice into which small cell lung carcinoma cells were implanted subcutaneous, it could be shown that an AAV infection which was carried out at the same time as a chemotherapy resulted in a faster recession of the tumors. Relapses occurred after a short time in both group.

- 15 However, in the AAV-infected chemotherapeutic group they responded better to another chemotherapy than those of the group only treated by means of chemotherapy. This shows that an AAV infection can reduce or even avoid the development of resistances.

- The use of the AAV viruses according to the invention can be made before, at the same
20 time with or after the chemotherapy or radiotherapy. However, it is carried out preferably after a first chemotherapeutic or radiotherapeutic treatment cycle.

In particular in the case of tumor kinds which per se show a poor response to a chemotherapy or radiotherapy it may be indicated to carry out the treatment before or together with the chemotherapy/radiotherapy to increase the efficiency of the therapy. By this the treatment doses can be lowered and therefore the side-effects can be reduced.

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The radiotherapy or chemotherapy may be any radiotherapy or chemotherapy which is adapted to the cancer to be treated. Such therapies have been known for years and along with the removal of the tumor by surgery they represent the established method of curing cancer diseases or increasing the life expectancy of a patient by some time. Therefore, a person skilled in the art is perfectly familiar with the measures of a radiotherapy or chemotherapy.

The use of AAV viruses according to the invention can be applied to any cancer kinds, the best success being expectable in connection with colon cancers, pancreatic carcinomas and brain tumors (in particular glioblastomas). The small cell lung carcinoma (SCLC) can preferably be treated therewith.

The application according to the invention is made intravenously, by means of infusions, intratumorally, orally (also by means of inhalations) or cutaneously. In this connection, the virus is formulated in a suitable preparation adapted to the pathway of administration. For an intravenous (also as an infusion) and intratumoral administration it is preferred to provide the virus in a physiological common salt solution, Ringer's solution or PBS

solution (phosphate-buffered salt solution), for a cutaneous administration it is preferred to provide it in the form of an ointment, suspension or gel, and for oral administration it is preferred to provide it in the form of an aerosol spray.

- 5 Depending on the patient's body weight the virus dose employed is 10^9 - 10^{10} AAV particles/kg body weight.

- A pharmaceutical composition is also provided according to the invention which in addition to the chemotherapeutic agent (cytostatic agent) contains adeno-associated viruses, in particular AAV-2. All chemotherapeutic agents (cytostatic agents) common in tumor therapy thus far can be used separately or in combination as a chemotherapeutic agent, e.g. cisplatin, etoposide, methothrexate, doxorubicin, cyclophosphamide, trofosfamide, busulfane, cytarabin, fluorouracil, mercaptopurins, vinblastinesulfate, vincristinesulfate, bleomycinsulfate or mitomycin. Thus, it is preferred for an intravenous (also by mean of infusion) and intratumoral administration to provide an injection solution, for a cutaneous administration to provide an ointment, for an oral administration to provide an aerosol spray. As a basis for the preparation of the infusion solution physiological common salt solution, Ringer's solution or PBS each are suitable in pure form or as a mixture. The amount of AAV depends on the patient's weight and is 10^9 - 10^{10} particles/kg body weight.
- 10 In the pharmaceutical composition it is contained in an amount suitable for an average body weight of 70 kg. The accurate dosage of the pharmaceutical composition according to the invention is determined by a physician and depends on the patient's sex and weight,
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- 20

severity of the disease, kind of administration and planned duration of administration. A composition according to the invention may also contain conventional auxiliary agents.

The common auxiliary agents such as carriers, binders, blasting agents, lubricants, solvents, solubilizers, release accelerators, release decelerators, emulsifiers, stabilizers, colorants of the taste correctives may be used as auxiliary agents.

The invention is explained in more detail by means of the attached figures.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a diagram of the protocol used for determining AAV-2-mediated drug-sensitization [sensibilization]. The proliferation of the SCLC cell lines after the infection and/or drug treatment was determined by the MTT assay (J Immunol. Methods, 1983, 56, pp. 55-63). The relative proliferation (A/Ao) was calculated by the ratio absorption of AAV-2-infected and/or drug-treated cells (A) to absorption of mock-infected untreated cells (Ao).

Figures 2a and b show the AAV-mediated [sensibilization] sensitization of the SCLC cell lines over cisplatin, the relative proliferation (A/Ao) of the SCLC cell lines, NCI-H209 (figure 2a) and NCI-H446 (figure 2b) following a mock infection (a: PBS alone; b: infected with a heat-inactivated gradient of Ad-2-infected cells) or infection with various multiplicities of an infection (MOI) with AAV-2 with (black [gray] columns) or without

(white columns) subsequent treatment with IC_{50} of cisplatin according to Table 1 (TCID, tissue culture infectious dose)

Figure 3 shows the AAV-mediated [sensibilization] sensitization of tumors in nude [naked] mice, derived from NCI-H209 cells (5 mice per group). The dose of cisplatin was 3 mg/kg body weight (weekly administration). The etoposide dose was 7.5 mg/kg body weight (administered three times a week). AAV-2 was administered weekly in a MOI of 10^8 TCID/animal. Arrows show the change of the treatment modalities: - interruption of the drug treatment and AAV-2 infection; + beginning of the treatment and the infection.

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DETAILED DESCRIPTION OF THE INVENTION

The invention is explained in more detail by way of the small cell lung carcinoma (SCLC).

This carcinoma type is generally characterized by an initially effective chemotherapy and

15 remission of the tumor. However, almost all of the patients suffer from a relapse which resulted from a resistance of the tumor cells to the first applied chemotherapy (usually cisplatin/etoposide). Therefore, it was tested in a model system with a human small-cell lung carcinoma cell line whether an infection with AAV enhances the cytotoxic effect of the chemotherapeutic agents in the cell culture and in tumors of immunodisturbed mice. It
20 is shown that the AAV infection increases significantly the effectiveness of the chemotherapy of SCLC tumor cells and SCLC tumors.

Example 1

[Sensibilization] Sensitization of SCLC cell lines over cisplatin and etoposide by AAV
infection

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Small cell lung carcinoma cell lines (NCI-H69, NCI-H164, NCI-H209, and NCI-H446) (Cancer Res., 1980, 40, 3502-3507; Cancer Res., 1985, 45, 2913-2923) were cultured in RPMI-1640 medium (Eurobio, Raunheim, Germany). HeLa cells were cultured in DMEN (Eurobio, Raunheim, Germany). All growth media were supplemented with glutamine 10 (Eurobio, 1%), antibiotics (penicillin and streptomycin) and 10% heat-inactivated fetal calf serum (PAA; Liz, Austria). The cultures were incubated at 37°C in a damp atmosphere with 5 % CO₂ and tested for mycoplasma contaminations at regular intervals.

The adeno-associated virus type 2 (AAV-2) was replicated in HeLa cells using, adenovirus 15 type 2 (Ad-2) as a helper. AAV was purified in a cesium-chloride gradient and titrated as described in J. Gen. Virol., 1994, 74, 2655-2662. The adenovirus type 2 inocula were clarified supernatants of Ad-2-infected HeLa cells.

The SCLC cells were suspended in PBS and incubated with purified AAV-2 in the 20 indicated multiplicities of infection (MOI). After 45 min at 37°C, unbound absorbed virus was removed by washing using PBS and the growth medium was supplemented. Either PBS or the heat-inactivated fraction (56°C, 30 min) of a CsCl gradient of Ad-2-infected

cells alone was used for the controls (mock infection), the density (1.14 g/cm^3) of the AAV-2-containing fraction which was used for the AAV-2 purification being indicated in the respective experiments. The volume/cell ratio of these experiments was 50 times greater ($5 \text{ ml}/16^6 \text{ cells}$) than the one used for the AAV infections.

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The AAV-2-infected or mock-infected cells were treated with cisplatin (Astra Medica, Frankfurt/Main, Germany) or etoposide (Bristol, Munich, Germany) or both in a ratio [cisplatin : etoposide = 1:2.5], the pharmacons dissolved in PBS, being added to the medium in the indicated concentration.

10.

The proliferation of the SCLC cells after the infection with AAV-2 and/or treatment with chemotherapeutic agents was determined by the modified MTT test (J. Immunol. Methods, 1983, 56, 55-63). After the infection or mock infection, the cells were placed in plates having 24 wells with a density of 10^5 cells/well and treated with the chemotherapeutic agents in the indicated concentrations. After six days, (NCI-H69, NCI-H446) or eight days (NCI-H146, NCI-H209), MTT, (3-(4, 5-dimethylthiazole-2-yl) -2, 5-diphenyltetrazoliumbromide; Sigma, Deisenhofen, Germany) was added to the culture up to a final concentration of 0.5 mg/ml . The cultures were then incubated at 37°C for 4 h to permit a reduction of MTT into blue formazan by mitochondrial dehydrogenases (Arch Biochem. Biophys., 1993, 303, 474-462), which indicates active proliferation of the cells.

20 The cells were centrifuged, washed with PBS, and formazan was solubilized in

isopropanol. The precipitated proteins were pelleted by centrifugation (1000 rpm, 15 min), and 200 μ l samples of the supernatant were measured to determine the optical density at 540 nm (OD540), OD690 being used as a reference and a Titertek Multiskan plus MKII densitometer being employed (Lab Systems, Finland).

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The relative proliferation (A/Ao) was defined as the ratio of the absorption (A) measured in the supernatant of AAV-2-infected and/or pharmacon-treated cells, compared with the absorption measured for the supernatant of the mock-infected cells and untreated control cells (Ao). The IC₅₀ value was defined as pharmacon concentrations resulting in a 50 %
10 inhibition of proliferation.

The AAV-2-infected cells (MOI values as indicated) or mock-infected cells were placed in plates having 24 wells and treated with cisplatin. After six or eight days, the relative proliferation was determined. In addition, kinetic studies were carried out to determine the
15 optimum treatment modalities for SCLC cells after an AAV infection. It was shown in these investigation that the AAV-mediated [sensibilization] sensitization reached a maximum after one to three hours following the infection. In this investigation the chemotherapeutic agents were administered three hours after the AAV infection. In order to exclude effects which are due to factors still present after the purification with the CsCl
20 gradient and the Ad-2 heat inactivation, a control, of the mock infection with PBS was carried out in addition to the mock infection with the fraction of the respective gradient of a cell lysate of Ad-2-infected cells.

The relative proliferation of NCI-H209-SCLC cells and NCI-H446-SCLC cells was measured after the infection with various multiples of the infectious units (MOI) of AAV-2 (10^2 - 10^5 tissue culture infectious dose (TCID) per cell) with and without subsequent treatment with cisplatin with the IC_{50} values listed in Table 1.

Table 1

Concentration of the chemotherapeutic agents which result in a 50 % inhibition of the proliferation (IC_{50}) of the SCLC cell lines

Cells Type	Cisplatin	Etoposide	Cisplatin/etoposide
NCI-H69	0.2	0.26	0.08/0.2
NCI-H146	0.11	0.025	0.008/0.02
NCI-H209	0.007	0.053	0.006/0.015
NCI-H446	0.15	0.21	0.042/0.105

As shown in Table 1, the SCLC cell lines NCI-H69 and NCI-H446 showed a high intrinsic resistance towards both pharmaceutical preparations. The susceptibility to cisplatin/etoposide treatment being lesser, whereas: NCI-H146 cells were highly susceptible to etoposide and the NCI-H209 cells were highly susceptible to both pharmacons.

As follows from figure 2 (a+b), the AAV-2 infection resulted in a decrease of the

proliferation rate of the cisplatin-treated cells with a MOI of 10^3 - 10^4 TCID/cell. The infection with a MOI of 10^5 TCID/cell resulted in no further increase. No significant inhibition of the proliferation was observed after the infection with lower MOI values of AAV-2 or after the mock infection, which indicates a specific effect due to the infection with high MOI of AAV-2. The relative proliferation of AAV-2-infected (10^{3-5} AAV/cell) and with cisplatin-treated (IC50) cells was lowered to 0.29 in NCI-H446 cells and to 0.25 in NCI-H209 cells as compared to the relative proliferation in (IC50) cells treated only with cisplatin (0.59 In NCI-H446 cells and 0.5 in NCI-H209 cells)

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Example 2

Quantification of the AAV-2-mediated pharmacon [sensibilization] sensitization of SCLC cell lines

In order to quantify the [sensibilization] sensitization of cells over chemotherapeutic agents after infection with AAV-2, dose-response curves were prepared. The relative proliferation of the cell lines was determined after a mock infection (PBS) or AAV infection with 10^3 or 10^4 TCID/cell and subsequent treatment with various concentrations of cisplatin or etoposide or a combination of both pharmaceutical preparations (Table 2).

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Table 2

[Sensibilization] Sensitization factor of SCLC cell lines which were treated with cisplatin

and/or etoposide and were infected with AAV.

Cell line	Chemotherapeutic agent	Sensitization factor (A/Ao)* 10 ⁴ Ip. AAV/Cell
NCI-H69	Cisplatin	1.43
	Etoposide	1.30
	Cisplatin/etoposide	1.45
NCI-H146	Cisplatin	1.37
	Etoposide	1.38
	Cisplatin/etoposide	1.33
NCI-H209	Cisplatin	2.33
	Etoposide	2.12
	cisplatin/etoposide	2.00
NCI-H446	Cisplatin	2.50
	Etoposide	3.00
	Cisplatin/etoposide	3.00

* The relative proliferation (A/Ao) was calculated by the ratio absorption of AAV-2-infected and/or pharmacon treated (A) cells to absorption of the mock-infected, untreated controls (Ao)

The [sensibilization] sensitization factors (SF) were defined as the ratio of the IC₅₀ values of infected cells compared with the IC₅₀ values of mock-infected cells. The [sensibilization] sensitization factor indicates the factor by which the concentration of a chemotherapeutic agent can be reduced after an infection with AAV-2 to obtain the same degree of proliferation inhibition. As summarized in Table 2 the [sensibilization] sensitization by AAV-2 in NCI-H69 and NCI-H146 was moderate (maximum SF about 1.4 with a MOI of 10⁴ TCID/cells) The infection of NCI-H209 or NCI-H446 induced a more

significant MOI-dependent [sensibilization] sensitization (maximum SF about 3 (NCI-H446) and 2.3 (NCI-H209) with a MOI of 10^4 TCID/cell). The AAV-2-mediated [sensibilization] sensitization did not depend on the chemotherapeutic agent employed.

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Example 3

AAV2-mediated pharmacon [sensibilization] sensitization of NCI-H209-derived tumors in
[naked] nude mice

10 H209 is a cell line which is derived from a tumor which was not treated
chemotherapeutically before cultured and is not resistant to drugs.

Female [naked] nude mice (CD1-nu/nu) from Iffa Credo (Brussels, Belgium) were kept in
isolators and were given water and food as desired. Experimentally growing SCLC cells
15 (H209) were injected subcutaneously into the side of the six-week-old mice (10^7 cells in
100 μ l PBS per animal). Five months after the inoculation of the cells, when the tumors
had reached an average volume of 200 mm³, the animals were infected weekly with AAV-
2 (intratumoral injection of 10^8 tissue culture infectious doses (TCID)) and/or with
chemotherapeutic agents by intraperitoneal injection of 3 mg/kg body weight cisplatin
20 (weekly) and 7.5 mg/kg body weight etoposide (three times a week). Details of the
beginning and end of the treatment are indicated in figure 3. In each group (control,
chemotherapeutic treatment, AAV2 infection, treatment + infection) five animals were

received. The infected and non-infected animals were kept in separate isolators. The tumor diameters were measured weekly and the tumor volume was determined by the formula tumor volume = $\frac{1}{2} \times \text{width} \times \text{depth} \times \text{height}$. The relative tumor volume (V/V_0) was determined for each animal and each time (ratio of the tumor volume $[V]$ compared
5 with the tumor at the beginning of the treatment ($[V_0]$).

As follows from figure 3, the treatment with chemotherapeutic agents resulted in a rapid decrease of the tumor volumes and a complete regression after three weeks of treatment. The combination of chemotherapy with AAV2 infection resulted in a more rapid decrease
10 of the tumor volume compared with animals which had only received a chemotherapy, which indicates a [sensibilization] sensitization of the pharmacon-treated tumor cells by AAV-2. The infection with AAV-2 alone had no significant effect, and the tumor volumes increased to the same extent as did the untreated controls. The treatment was discontinued after complete regression of the tumors and was resumed in the case of a relapse. The
15 treatment of relapses was less effective with animals, which had only received drug treatment, as compared to the animals infected with AAV and treated chemotherapeutically. This shows the development of a resistance to the initial treatment at least in the chemotherapeutically treated animal group. The relapses in AAV-2-treated animals were still susceptible to cisplatin and etoposide treatment but the tumor regression
20 was slower as compared to the regression of the initial tumors. In 3 of 5 AAV-2-infected animals the tumors regressed completely in week 9, in contrast to the tumors of animals which were only treated with chemotherapeutic agents, a complete regression of the tumor

not being induced.

Claims

1) Use of adeno-associated viruses for lowering the radiotherapy-induced or chemotherapy-induced resistance in patients who suffer from a cancer which is to be treated by radiotherapy or chemotherapy.

5

2) Use according to claim 1, wherein the adeno-associated viruses are used in a dose of 10^9 - 10^{10} AAV particles/kg body weight.

3) Use according to any of claims 1 to 2, wherein the adeno-associated virus is adeno-associated virus 2 (AAV-2).

10

4) Use according to any of claims 1 to 3, wherein the cancer to be treated by radiotherapy or chemotherapy is a colon cancer, pancreatic carcinoma or brain tumor or small cell lung carcinoma.

15

5) Use according to any of claims 1 to 4, wherein the use is made intravenously, cutaneously, orally or intratumorally.

6) Use according to any of claims 1 to 5, wherein the use is made before, after or simultaneously with a chemotherapy or radiotherapy.

20

7) A pharmaceutical composition containing a chemotherapeutic agent and adeno-

associated viruses.

8.) The pharmaceutical composition according to claim 7, wherein the chemotherapeutic agent is cisplatin and/or etoposide.

5

9) The pharmaceutical composition according to claim 7 or 8, wherein it is an injection or infusion solution, an aerosol spray or an ointment.

ABSTRACT OF THE DISCLOSURE

The invention relates to the use of adeno-associated viruses for decreasing the radiotherapy-induced or chemotherapy-induced resistance in patients who suffer from a
5 cancer which is to be treated by radiotherapy or chemotherapy.

Mouse

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The mouse has played a key role in making many medical research advances possible. Mice age 30 times more rapidly than human beings. This short life span makes them ideal for studies on aging, for example.

Specially bred animals, such as the **"nude" mouse**, which has unusually low immunity, help us to understand tumors and treatments for cancer. The many strains of inbred mice with natural genetic deficiencies have resulted in a wide range of genetic models of human diseases. Researchers can obtain a strain of mouse with a disease affecting almost any organ system or tissue to mimic the human disease they would like to study.

Mice reproduce readily and plentifully. Because of this, researchers can use them to study genetics, as well as the abnormalities of fetal development associated with poor genetic background, or the ways in which addictive drugs taken by pregnant females affect offspring.

Contributions of mice to the knowledge of basic genetics and immunology have resulted in the development of organ transplantation.

Vaccines for many diseases, including **whooping cough** and **yellow fever**, were developed and tested in mice. Today, surgical techniques have improved so much that even **heart transplantation** is being studied in the mouse. These animals contribute to research on **cancer**, **heart disease**, **deafness**, **epilepsy**, **muscular dystrophy**, **eye abnormalities**, **brain dysfunction**, **nerve disorders**, **blood clotting disorders**, **immune diseases**, *in vitro* fertilization, **Hodgkin's disease**... the list is almost endless.

The "Nude" Mouse



The hairless mouse is uniquely valuable to cancer research. In 1962, when the first hairless mouse turned up among experimental animals at a laboratory in Scotland, no one knew what to make of it.

Then tests on its descendants led to a surprising discovery. Cells from certain human tumors, implanted in these mice, continued to grow. For the first time, scientists could study a human cancer cell in another animal, without risking a human life.

As it turned out, what makes the mouse a perfect host also makes it bald. It is born without a thymus gland, which would regulate its defenses against disease, and the same genetic accident that deprived it of the gland also destroyed its ability to grow hair. With a virtually limitless supply of these mice, researchers have been able to test an array of new drugs and treatments; and doctors are designing treatment plans by trying alternative therapies on a series of nude mice.

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Mice and Research

Along with rats and other rodents, mice make up the majority of animals used for medical research. Their small size, low cost and ease in handling make them ideal for laboratory experiments. In addition, scientists can breed different strains of mice with natural genetic deficiencies to achieve models of human diseases. The following are fields of medicine and research in which the use of mice has advanced understanding and shown potential for cures.

CANCER:

Through breeding, scientists have developed mice with leukemia, breast cancer and many other types of cancer, allowing new treatments to be tested on animal models instead of humans.

- Scientists have discovered that cells from certain human tumors could be placed into immunologically deficient ("nude") mice and would not be rejected. This allows the study of human cancer cells without risking human lives.
- Studies with mice have shown that the immune system can be stimulated by genetically altered tumor cells, leading to hopes that this "gene therapy" technique can be used to fight cancer in humans. (1)

GENETICS:

Much is known about the genetic makeup of the mouse; it is probably the most commonly used mammal in genetic research. Scientists genetically engineer mice and breed them so that they develop human diseases that can then be studied or produce chemicals that can be used for the treatment of diseases. (2)

IMMUNOLOGY:

Much of our knowledge about the immune system has come from studies in mice.

Scientists are waging a continuous battle in the search for the best animal model for the study of AIDS, and much has been accomplished from research done with mice. Researchers have transplanted cells of the human immune system into SCID (Severe Combined Immuno-Deficient) mice, which lack their own immune systems. As a result, mice have dramatically increased their life spans. Experiments such as this have enabled scientists to apply information gained from mice to humans suffering from AIDS.

AGING:

Because mice age much faster than humans, they are ideal for the study of human aging.

Researchers have found that immune system effectiveness and the ovaries decline with age similarly in mice and humans. This finding allows scientists to study mice in order to improve immune response in the elderly, reduce their chances of developing diseases and understand the

decrease in their reproductive organ function.

PRODUCT SAFETY:

Mice, along with other rodents, are used in product safety tests such as the repeated-dose, chronic-toxicity test - which measures the effects of long-term exposure to a product, or the developmental and reproductive toxicity test - which measures the likelihood of infertility or effects on pregnancy because of product usage.

VIROLOGY:

Scientists have used specially-bred mice as hosts for viruses, infectious agents that require living cells in order to multiply and survive.

- Research with mice helped develop vaccines to counter influenza, polio, yellow fever and rabies.
- Research on mice has shown that the host as well as the agent plays a major role in viral infections.

TRANSFER OF EMBRYOS:

The successful transfer of human embryos is possible today because of experiments done with mouse embryos. This technique can be used to improve reproduction in domestic and endangered species.

RADIATION EXPOSURE:

The research of inbred strains of mice is instrumental in understanding the harmful effects of radiation exposure and developing treatments.

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RODENTS AS MODELS FOR BIOMEDICAL RESEARCH

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MICE



INTRODUCTION

The house mouse, *Mus musculus*, has a name which originally meant to *steal*, but despite its propensity for petty thievery, it has enjoyed a far better reputation than its fellow rodent, the rat. The mouse, once honored in ancient coins, writings, and paintings, has the dubious distinction of being employed in research studies beginning in the early 1600s.

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II. USES IN RESEARCH

Mice represent the primary species used in research, comprising 67% of all animals used in biomedical research and testing. Their short life span, proclivity for reproduction, known genetic background, and minimal expense for purchase and maintenance have made them a desirable animal model. Mice are used in studies involving aging, behavioral research, bioassay and pharmacological screening, chemical mutagenesis and carcinogenesis, convulsive disorders, diabetes and obesity, embryology, immunology, infectious disease research (bacterial, fungal, parasitic, viral), naturally occurring neoplasias (hematopoietic system, mammary tissue, male and female reproductive systems, urinary, endocrine, respiratory, digestive, musculoskeletal, nervous, cardiovascular, and integumentary systems), and ophthalmic research. Mice have been used for monoclonal antibody production, producing greater concentrations of antibodies with less labor and expense than could be obtained from polyclonal generation in larger species such as the rabbit, goat, or sheep.

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Immunodeficient Rodents

Immunodeficient rodents are indispensable research models for biomedical investigators for studies in oncology, immunology, and infectious diseases. Today, biomedical researchers use a number of naturally occurring and transgenic strains of immunodeficient mice and rats to study the immune system, rejection of tissue transplants, infections, cancer and tumor growth.

With the development of "knockout" immunodeficient mice, in which genes affecting the immune system are inactivated in the research animal, new fields of research are being opened to precisely study the role of selected components of the immune system. The recent flurry of advances in designing research animals -- including models with multiple immunocompromised functions or genetic deficiencies -- began some 20 years after the discovery of mice with a single, naturally occurring immunodeficiency.

In the early days of immune function research, observers noted that all animals have the physiological ability to "self-discriminate." That is, the body can discriminate between its own cells and those of another animal -- even one of the same species -- and then launch an immune response against foreign cells or substances. Early researchers also noted that blood cells called lymphocytes appeared to play a key role in

the immune response.

Like other blood cells, lymphocytes differentiate from pluripotent stem cells in bone marrow. Lymphocytes that continue their maturation in bone marrow develop into B cells, while those that migrate to the thymus and complete maturation there become T cells. Mature B cells and T cells are most concentrated in lymph nodes, the spleen, and other lymphatic organs where the lymphocytes are most likely to encounter antigens -- foreign substances that evoke the production of antibodies and cytotoxic cellular responses.

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T, B and NK Cell Functions

Both B and T cells are able to recognize antigens. B cells are responsible for *humoral, or serum, immunity* by producing immunoglobulins, or Igs. These Igs are divided into five chief classes -- IgG, IgM, IgA, IgD, and IgE -- each with special properties. T cells, making up about 70 percent of all lymphocytes, are responsible for *cellular immunity*, meaning they attack and kill antigens directly. T cells do not themselves make antibodies but they help regulate the production of antibodies by the B cells. There are four types of T lymphocytes -- helper, cytotoxic, delayed hypersensitivity (associated with allergies) and suppressor.

Differentiation of B and T cells into a vast variety of cloned cell types, each responding to a specific antigen, involves two phases -- the primary or antigen-independent phase and the secondary or antigen-dependent phase. During the primary phase, stem cells proceed through stages of differentiation to generate vast amounts of B or T cell clones, each with unique antigen receptors. The immune system generates an incredibly diverse range of gene sequences, or antigen-binding specificities for antibodies.

The secondary (antigen-dependent) phase involves only B cells, which can recognize an infinite number of antigens (but each individual B cell recognizes only one antigen). When a particular antigen binds to the antigen receptors on the appropriate B cell, that B cell is triggered to proliferate into a large clone of cells, all responsive to the specific antigen. In this *clonal selection* process, some of the cloned B cells become long-lived memory cells and others differentiate into plasma cells secreting antibodies.

Another type of immune cell was discovered in 1975 -- the natural killer (NK) cell, which looks like a lymphocyte but contains granules resembling granulocytes. NK cells apparently recognize some feature of the target cells, either directly or via receptors that attach to the tails of antibodies on the target cell's surface. As a result, NK cells act by releasing the contents of their granules to kill the target cells or by recruiting the help of other immune cells.

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Immunodeficiency Mutations In Mice



More than 4,000 genes of the mouse have now been assigned to specific chromosomal locations. Many genes were first identified following spontaneous mutations that produced distinctive physical characteristics. Single-gene mouse mutants have provided highly useful experimental research models. Two key single-gene, naturally occurring mutations are the nude (*nu*) and the severe combined immunodeficiency (*SCID*); both are important research models for the study of xenografts, transplanted tissues and tumors from foreign species. Other single-gene mutations commonly used as research models include the beige (*bg*) and the X-linked immune deficiency (*xid*).

The *nu* mutation was first reported in 1966 in a closed stock of mice in a laboratory in Glasgow, Scotland. It was not until 1968, however, that it was discovered that the homozygous nude mouse also lacked a functional thymus, i.e., it was *athymic*. The mutation produces a hairless state, generating the name "nude." The other, unique defect of nude mice is the failure of the thymus to develop normally to maturity. The thymus remains rudimentary and produces reduced numbers of mature T cells. This means nude homozygotes (animals with identical mutant genes at corresponding chromosome loci) do not reject allografts and often do not reject xenografts (tissue from another species). The discovery that human neoplasms (tumors) could be grown in nude mice was immediately recognized as an important research tool. Thus, the spontaneous mutation of *nu* among laboratory mice was a serendipitous development that led to the nude mouse becoming the first animal model of a severe immunodeficiency. In the decades since, the nude mouse has been widely utilized by researchers studying factors regulating transplantable human tumor growth and cancer metastasis.

Although it lacks T cells, the nude mouse has a normal complement of bone-marrow-dependent B cells. It thus presented a unique tool for the study of the role of the thymus on lymphocyte differentiation, investigations of B cell functions (including interactions with other immune cells) and studies of the actions of other immune cells, including the natural killer (NK) cells. Nude mice have elevated levels of both macrophages and NK cells; their macrophages are more potent than those from mice with a normal thymus.

The first successful transplantation of a human malignant tumor to nude mice was reported in 1969. Nude mice have been used extensively in studies of the tumorigenicity of *in vitro* cultured cells. Nude mice are also widely utilized in evaluating anticancer agents prior to human clinical trials.

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SCID Mice: Accident of Nature

Serendipity also played a role in the discovery of another important mutant strain of immunodeficient mice, which lacks both B and T cells, called severe combined immunodeficiency (SCID) mice. During routine lab tests on the immune system in mice, Dr. Melvin J. Bosma of the Fox Chase Cancer Center in Philadelphia discovered the strain in 1980. The first SCID mice were an accident of nature, the product of chance matings of apparently normal mice that carried a recessive mutant gene now called *SCID*. Some of the offspring inherited a complete pair of *SCID* genes and were born with the *SCID* defect.

"We were conducting antibody studies when we found that some of these mice lacked antibody," recalled Dr. Bosma. "The disease seemed to affect cellular immunity, too. These animals had tiny lymph nodes and the thymus was about one-tenth normal size. It took about 3 years to determine and demonstrate that these mice had a severe immune deficiency disease similar to that called SCID that afflicts some human children."

Dr. Bosma's laboratory bred these mice with each other to produce the original SCID colony. At first, the SCID mouse attracted interest because it was the first known animal model for human SCID, a congenital syndrome that is usually fatal to human babies. The SCID mouse is also an excellent model for studying the relationship between immune defects and cancers of the lymph system. The Fox Chase researchers found that histologic abnormalities in SCID were remarkably uniform, because they all share the same underlying genetic defect. Dr. Bosma and his colleagues also noted that, like nude mice, the normal immune function of SCID mice could be genetically reconstituted by "seeding" with lymphocytes from bone marrow of normal mice. But because the SCID model lacks both B and T cells, it presents much greater potential for studies of selective reconstitution of immune cell populations.

The action of the *SCID* mutation in blocking lymphocyte development is not absolute, however. As they mature, some adult *SCID* mice generate a few clones of functional B and T cells. These *SCID* mice are said to be "leaky," meaning that low levels of B and T cells are detectable. "By 10 to 14 months of age, virtually all [C.B-17] *SCID* mice are leaky," says Dr. Bosma. "Those with detectable B cells also invariably contain T cells. This implies that the development and growth in numbers of B cells in *SCID* mice may be totally dependent on T cells and -- perhaps -- vice versa."

However, other researchers now report that another strain of *SCID* mice appears to be virtually devoid of the leaky phenotype, the ICR *SCID* mouse.

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Beige and *Xid* Mutations

Two other single-gene immunodeficient mouse models have less severely compromised immune systems than the nude and *SCID* models. These are models with the beige (*bg*) and the X-linked immunodeficiency (*xid*) mutations. Mice with the X-linked recessive mutation have been widely used in studies of B cell development and maturation. Homozygous *xid* females and *xid* males do not respond to thymus-independent antigens and also fail to respond to specific thymus-dependent antigens. No defects in T cell functions, such as graft rejection, are noted in *xid* mice.

The beige model, named for its hair color, has been used extensively for studies of selective NK cell deficiency. A defect in the NK-cell function in *bg/bg* mice blocks the normal process of degranulation, leading to impaired antibody-dependent and antibody-independent cytolysis of tumor cells. Mice with the *bg* mutation show greater-than-normal susceptibility to infection by pyrogenic (fever-producing) bacteria. Beige mice have been used in studies of hematopoietic cell differentiation.

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Multiple-Gene Immunodeficiencies

Researchers have been able to intercross various immunodeficient mice models to selectively investigate the effects of combined mutations. For example, mice that are bred to be simultaneously homozygous for both *xid* and *nu* mutations are severely deficient in both B cells and T cells. But thymus grafts in these doubly homozygous mice promote B cell development, indicating that the maturation of *xid*-mediated B cells past the early developmental stages is T cell-dependent.

The ability to selectively breed mice models combining various immune deficiency mutations is invaluable to researchers. A prominent example is the triple-deficient (*bg-nu-xid*) model. By controlling the inheritance of the three mutations, researchers can devise experimental systems designed to bear the effects resulting from any single mutation or in various combinations of the mutations in the same experiment.

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Importance of Genetic Background

When working with a specific immunological mutation in rodents, it is important to select the appropriate genetic background (stock or strain of mouse) upon which it should be maintained. Experienced researchers say the ideal choice for mutant maintenance is an inbred strain. Once the mutant gene is established on a selected inbred strain through several generations of backcrossing (generally 10 or more), the resulting

offspring are considered a *congenic* strain -- a strain that is genetically identical to its confrere inbred strain except for the mutant gene (and, perhaps, closely linked genes). By comparing the immunodeficient congenic strain with the partner inbred strain, investigators can study the functions demonstrated by the gene in question.

The Swiss nude immunodeficient strain is a general purpose outbred model that is economical and easy to maintain. The C57BL/6 nude is also a general purpose strain suitable for a wide range of studies requiring an immunodeficient research animal. The investigator can select an inbred model, in which all animals are genetically identical, or an outbred model, which has animals representing a diverse gene pool. Outbred models, such as the Swiss nude, are more economical to produce because Swiss females have good nurturing instincts and abilities, thus producing larger litters with more robust pups.

The double-mutant C.B-17-scid-beige model is deficient in B, T and NK cells, making it valuable for cancer research because one has removed another layer of immunity -- the [NK] population of cells that kill tumors. Another immunodeficient model, the athymic (nude) rat has the same (or very similar) *nu* mutation as the nude mouse, but because of the rat's larger size it is a better research model for investigations requiring extensive surgery.

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SCID-hu Mouse Chimera Has Functioning Human Immune Cells

Soon after biomedical researchers began working with immunodeficient mice models in the 1960s, some scientists wondered whether these mice -- which readily accepted human cancer tumor transplants -- could someday accept transplants from the human immune system to replace their own crippled immune systems. Such a mouse model of the human immune system would be a momentous step forward in using laboratory animals as realistic living models for investigations of human diseases, including AIDS.

In September of 1988 two groups of researchers almost simultaneously announced that they had succeeded in transplanting elements of the human immune system into SCID mice. They had used totally different approaches in creating their human-mouse chimeras.

At Stanford University, Dr. J. Mike McCune and Dr. Irving Weissman implanted 300 SCID mice with tissue taken from human fetal thymus. In some of the animals they also transplanted human fetal tissue from the liver and lymph nodes, along with the thymus tissue, into the kidneys of the SCID mice. In fetal development of mammals, stem cells that will eventually become part of bone marrow are initially produced in the liver. Progenitor T cells originate in the thymus and then enter the lymph nodes as mature and functioning T cells.

The implanted human fetal tissues soon produced mature human T cells in the Stanford SCID mice. The mice that received fetal lymph tissue also developed mature human B cells. The chimera, named the SCID-hu, immediately proved useful in studies of drug toxicity and efficacy that would not be appropriate in human subjects because of uncertainties about safety in early stages of testing. Dr. McCune showed in 1989 that the AIDS drug AZT suppressed HIV replication in SCID-hu mice. His study, requiring only a few weeks in mice, produced results similar to others that took 5 years of clinical trials with human AIDS patients.

Another team of researchers, at the Medical Biology Institute in La Jolla, Calif., reported they also used SCID mice as hosts of human immune transplants. But the La Jolla researchers, headed by Dr. Donald Mosier,

used a simpler approach. They injected human peripheral blood leukocytes (PBLs) into the mice. Human PBLs contain B and T cells. Almost immediately after injection, the mice began replicating the human B and T cells.

This chimera, called the hu-PBL-SCID, also was able to produce human tetanus antibodies when injected with tetanus toxin, further demonstrating that its immune system was functioning as though it was naturally human.

This human-mouse chimera, the SCID-hu, dramatically demonstrates sciences' ability to create mouse models that are more precise analogs of the human condition. It offers great potential for a broad range of research with human applications, including the study of HIV and other viral infections, immune system development, congenital diseases and other diseases.

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Opportunities in Transgenic Technology

Interest in immunodeficient animals has mushroomed in recent years with advances in molecular biology and genetics that allow researchers to manipulate the genome of mice to eliminate or add genes and even replace selected genes. Science can actually engineer laboratory mice to meet specific research needs and protocols. Creating new, genetically engineered animal research models involves two transgenic techniques — **microinjection** of cloned genes randomly inserted into the host DNA and **gene targeting** or homologous recombination between cloned DNA and one of the identical copies of the sequence normally present in the chromosome. The more complete names of these two transgenic techniques are:

- ✱ **classical pronuclear microinjection:** introduction of foreign DNA into embryonic pronuclei resulting in random integration and expression and
- ✱ **embryonic stem (ES) cell-mediated gene targeting:** introduction of genetically modified ES cells into recipient embryos resulting in the ablation (knockout) or modification of a specific genetic expression.

Transgenic animals are designed to exhibit either a *gain of function* (expression of a novel cell-surface receptor) or a *loss of function* (knockout of a cellular function). Classical pronuclear microinjection techniques have been used for 15 years to create mouse models which express unique phenotypes. The major flaw in the pronuclear microinjection models has been the random nature of transgene integration locus and copy number. Expression patterns may vary significantly in a series of lines expressing identical transgenes. Modifiers of expression such as age, sex and health status further confound the process, increasing potential for variability.

By using ES cell gene knockout technology, an investigator can produce an animal model in which expression (or the lack of expression) is highly predictable. A clone of cultured ES cells is selected in which a specific DNA sequence in the mouse genome has been modified (usually inactivated). Transformation of cultured cells with foreign DNA is relatively simple and most commonly is achieved using a procedure called electroporation. All transgenic models, whether targeted or untargeted, still may present unpredictable expression patterns due to incomplete knockout of the targeted gene, redundancy within the genome or unanticipated genetic interactions, such as down-regulation of other genes.

Despite some unpredictability questions, transgenic knockout technology can produce research animals that are "custom designed" to meet the specific needs of an investigator's experimental protocol. Knockout technology, or homologous recombination, is also a valuable tool for determining functions of specific genes. Dr. Mario R. Capecchi of the University of Utah School of Medicine, one of the pioneers of gene

targeting, explains the concept of targeted gene replacement:

- ✱ If we suspected a particular gene were involved in brain development, we could generate mouse embryos in which the normal gene was "knocked out" -- that is, completely inactivated. If this inactivation caused newborn mice to have a malformed cerebellum, we would know that the gene in question was essential to forming that part of the brain.

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Transgenic Animals Are 'Extraordinary'

Transgenic technologies, which are rapidly expanding, provide potential advantages over naturally occurring mutations in developing animals specifically tailored to mimic elements of the human system or human diseases. The knockout technique has already produced mouse models of many human diseases, including retinoblastoma, p53 antioncogene deficiency, Gaucher's disease and others.

Knockout mice may display the most severe types of human inherited disorders because they lack expression from the corresponding genes of the mouse. Some knockout models are particularly fascinating because they live with inactivated genes that formerly were believed to be essential for survival. The expanding ability to produce double (and multiple) gene knockout animal models further enhances investigators' abilities to elucidate the functions of specific combinations of genes and to more accurately model features of the human immune system and human diseases.

In only about 10 years, genetically engineered mice have brought dramatic and exciting advances to biomedical research. "These mice are, quite simply, extraordinary," says Dr. Joseph Perch, vice president for grants and special programs at the Howard Hughes Medical Institute in Chevy Chase, MD.

Due to rapid advances in genetic engineering and increasing use of the technology by laboratories, it is evident that these extraordinary models will lead to further significant developments in knowledge of the immune system's broad array of components and their complex functions. Obviously, transgenic animal research models will contribute to important advances in a wide range of biomedical research.

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Social and Ethical Concerns - Safety

The advent of biotechnology, more so than any other technological innovation, has made it clear to scientists that they can no longer dismiss ethical concerns in society as irrelevant to the scientific enterprise, on the grounds that, by its very nature, science is and ought to be "value-free" or "value-neutral". More pragmatically, it is evident that society is extremely exercised about the genetic engineering of animals, and that a failure on the part of scientists to enter into the dialogue concerning the ethical issues raised by transgenics is likely to leave the development of principles governing the field to those who shout the loudest.

In the first place, it is necessary to separate out genuine moral issues from spurious ones, which nonetheless command and galvanize a great deal of public attention. For example, religious groups complain that creation of transgenic animals violates divine law, or fails to show proper respect for life. Other critics argue that humans were not "meant" to create life. Still others see species as inviolable, or assert that humans

should not alter "nature". Such objections have stirred public skepticism and fear regarding biotechnology. When scientists fail to exhaustively discuss - and deal with - the genuine moral issues growing out of genetic engineering of animals, one is inexorably led to a version of Gresham's Law, wherein, instead of bad money driving good money out of circulation, bad (or spurious) ethical and social concerns drive genuine and legitimate ethical and social concerns out of the social mind. Thus it is imperative that researchers planning to develop or use transgenic animals be fully cognizant of, and prepared to address, the genuine social and moral questions raised by such activities, lest the sensationalistic, baseless concerns inform and shape the public response to biotechnology.

The first genuine bioethical issue relevant to the creation and use of transgenic animals in biomedical research concerns the possible danger to humans and/or other animals which might be presented by the animals in question.

Perhaps the most dramatic real case of such a concern is exemplified by the mouse model for AIDS created at the National Institute of Allergy and Infectious Disease of the National Institutes of Health. HIV-1, the pathogen causing human AIDS, naturally infects only humans and chimpanzees, and chimpanzees remain asymptomatic. In an attempt to create an "animal model" for the disease, researchers introduced the HIV genome into mouse embryos by microinjection and propagated these mice by breeding. Although the purpose of the model was primarily to study viral latency, F₁ progeny resulting from the mating of one of the founder animals, its nontransgenic mates developed symptoms similar in some respects to human AIDS. Furthermore, the tissues of these animals produced infectious HIV particles.

Obviously, the creation of mice capable of harboring infectious HIV virus represents a genuine and legitimate social concern of both a moral and prudential nature regarding biosafety. The moral question is, of course, whether one ought to produce such a potentially dangerous organism. The prudential question is, given the decision that the benefit outweighs the risk, and therefore that such an animal should be created (leaving aside separate moral questions regarding animal suffering), how does one reduce the risk to an acceptable minimum?

These sorts of concerns are legitimate and understandable and encapsulate questions which any researcher planning to develop or utilize transgenic organisms should carefully address. Indeed, potential dangers from a transgenic organism may be far more subtle than just transmission of the disease it is designed to host. For example, if one were genetically engineering for resistance to a given pathogen in an animal, one could conceivably be selecting new variants among the natural mutations of that microbe to which the modified animal would not be resistant. One possible example of this sort of reaction has recently been discussed above regarding the SCID-hu mouse developed as a model for AIDS. These animals are genetically engineered to possess a human immune system, and are then infected with the AIDS virus. Some researchers suggest that, in such a mouse, the AIDS virus could become more virulent and infectious through new routes of transmission in virtue of interacting with native mouse viruses. This could conceivably wreak havoc both with animals and humans. Genetically engineered animals could conceivably damage extant ecosystems if such animals are not confined. Thus, it would seem morally incumbent both to do careful cost-benefit analysis before creating any transgenic animal, and also to build in an additional safety mechanism by demanding significant balance of benefit over cost to compensate, at least in some measure, for unknown or unrecognized dangers. Michael Crichton's recent novel, *Jurassic Park*, provides an ingenious, scientifically based, albeit fanciful account of how small, unanticipated problems in genetic engineering can amplify into major catastrophes along lines describable by the mathematical theory of chaos.

Thus the first ethical requirement relevant to creating transgenic animals is making a case that benefits clearly outweigh risks, with significant allowance made for unforeseeable risk. If this demand is met, the next ethical requirement is the demand that one control for the remaining risks.

Fortunately, the research community has been extremely sensitive to the theoretical dimensions of laboratory biosafety. Researchers utilizing transgenic animals for disease-related study should familiarize themselves with the principles encoded in the CDC-NIH publications *Guidelines for Research Involving Recombinant DNA Molecules and Biosafety in Microbiological and Biomedical Laboratories* and *Biosafety in Microbiological and Biomedical Laboratories*. These volumes describe four increasingly stringent levels of biological containment (Biosafety levels 1 to 4).

In the case of the AIDS mice mentioned above, the microinjected embryos were inserted into surrogate female mice, which were transferred to a stainless steel glove box within a BL4 facility. All transgenic animals were maintained in the glove box closed system for the duration of the experiment. Indeed, one can characterize the containment procedures for this experiment as BL4+, for, in addition to standard BL4 procedures, the containment boxes were surrounded by bleach-filled moats and traps to provide "overkill" assurances that the danger was contained.

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RATS



INTRODUCTION

Rats have been regarded as vile, insidious vermin, damned for their role in the Black Plague of the Middle Ages, and for the injury and destruction they have caused to humans and property throughout the ages. However, one species of rat, *Rattus norvegicus*, has been used in research since the mid 1800s, in what Lindsey described as an ascendancy from the gutter to a place of nobility through its contributions to human health and well-being. In an ever evolving process since the first crude uses of rats in research, investigators have sought, through trial and error, to develop appropriate husbandry, care, and use techniques to minimize or eliminate the impact of variables such as nutrition and disease on research results. Early ignorance of basic needs translated into inappropriate or substandard care, which affected the health and well-being of the animals first and foremost, but also affected the quality and reliability of the research data that was generated. Advances were made in understanding the requirements for and provision of adequate nutrition, in recognition and control/elimination of latent infectious diseases, and in providing sanitary environments to minimize contamination of and disease in the rats.

dogs, cats, pigs, and certain primate species; however, there are over 41,700 species of vertebrates. The potential for any of these species to be used as research subjects increases as our knowledge of physiological and ecological processes expands.

Examples of the use of nontraditional laboratory animals in biomedical research are common. Woodchucks (*Marmota monax*) are used as models to study obesity, energy balance, hepatitis, and hepatocellular carcinomas and the opossum (*Didelphis virginiana*) as a model for endocarditis.

Field and laboratory research on a wide variety of species is conducted to learn more about the species and its biology and ecology. Many studies are conducted in the natural environment where they are found, and others bring animals into the laboratory for study. Scientific societies in North America have developed guidelines for conducting research on the species of their concern. Field methods for mammals have been published by the American Society of Mammalogists. The Wildlife Society includes field research guidelines in its *Research and Management Techniques for Wildlife and Habitats*. The Canadian Council on Animal Care and the Universities Federation for Animal Welfare have published manuals that include guidelines for a wide range of species. The U.S. government has adopted "U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training".

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TABLE 1. Rodent Animal Models of Human Disease

Animal Group	Biomedical Problem	Specific Disease
Mouse	Genetic/developmental defect	Anemia, hereditary Athymic Autosomal trisomies Chediak-Higashi syndrome Copper malabsorption, X-linked Exencephaly Hereditary asplenia L cell mutant Megacolon, aganglionic Megaloblastic anemia Polycystic kidney disease Testicular feminization
Mouse	Neoplastic disease	Adenocarcinoma, DES Adenoma, salivary Angiosarcoma, liver Carcinoma, cervix Carcinoma, embryonal Hodgkin's disease Leukemia, myelogenous Malignant tumor transplant Mammary tumor Ovarian tumor Preneoplastic lymphoid hyperplasia

		Teratoma and teratocarcinoma
Mouse	Metabolic/ nutritional disease	Amyloidosis Diabetes mellitus Gammopathies, monoclonal Gestational diabetes Globoid cell leukodystrophy Glucose-6-phosphate dehydrogenase deficiency Histidinemia Hypervitaminosis A Hypophosphatemia (rickets) Mast cell deficiency Methylmercury poisoning Niemann-Pick Disease Nonobese diabetic Ochratoxicosis ornithine transcarbamylase deficiency Paraproteinemia, idiopathic Thalassemia, alpha
Mouse	Degenerative Disease	Adenosis, vagina/cervix Autoimmune disease Biliary obstruction Diverticulosis, oviduct Dysbaric osteonecrosis Proliferative glomerulonephritis Immunosuppression Macroglobulinemic neuropathy Menkes's disease Motor neuron disease Pulmonary fibrosis, bleomycin Pulmonary fibrosis, solvents/oxygen Reye's syndrome Salpingitis Vitiligo
Mouse	Infectious disease	Avian reovirus <i>Capillaria hepatica</i> Cytomegalovirus Encephalomyocarditis Giardiasis Hepatitis, reovirus Influenza B Lymphocytic choriomeningitis Meningoencephalitis, amoebic Meningoencephalitis, <i>Angiostrongylus</i> Scrapie Theiler's encephalomyelitis Trypanosomiasis



NIH NEWS RELEASE

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Allergy and Infectious DiseaseFOR IMMEDIATE RELEASE
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Transgenic Mice Aid Research into Deadly Cancer

Scientists will be better able to fight Burkitt's lymphoma, a rare but deadly cancer that attacks children and AIDS patients, now that they have achieved a decades-long goal of genetically engineering mice to develop the disease. These transgenic mice are a powerful new tool researchers will use to understand the molecular and genetic components of this cancer.

Previous attempts to engineer such "Burkitt's mice" failed, but a team of scientists at the National Institutes of Health (NIH) finally came up with the right genetic mix. "We in effect created a 'mini-gene' that reproduces the cancer as it occurs in people," says Herbert C. Morse III, M.D., chief of the immunopathology lab at the National Institute of Allergy and Infectious Diseases (NIAID). The results of this work, supported by NIAID and the National Cancer Institute (NCI), are detailed in the current issue of the *Journal of Experimental Medicine*.

"This new animal model may allow researchers to find ways to treat Burkitt's patients who don't respond to the standard treatment," says Dr. Morse. "It will also help us understand why the cells 'go bad' to cause this malignancy." Scientists still don't know all the factors that contribute to Burkitt's lymphoma in people, despite having created Burkitt's mice.

Scientists *do* know that Burkitt's lymphoma arises in the immune system, and that the groundwork for the cancer is laid when a gene called MYC (pronounced "mick") is accidentally moved from its usual spot on chromosome 8 to a new location, often on chromosome 14. Normally, the MYC gene, which stimulates cell growth, is strictly controlled by nearby regulator genes on chromosome 8. But when MYC slips away to a different chromosome, it also evades its controls. Under the looser supervision of regulatory genes that govern the immune system, MYC "goes ripping out of control," Dr. Morse explains, causing runaway growth of the immune system's B cells, which in turn leads to tumors called lymphomas.

But scientists have much more to learn about how other genetic mutations, environmental factors and infectious agents, such as the Epstein-Barr virus, work together to cause Burkitt's lymphoma. In fact, studying the Burkitt's mice may lead to new understandings about why some people are predisposed to Burkitt's lymphoma, while other people are predisposed to other kinds of cancers, says Siegfried Janz, M.D., of NCI. "The only way to find this out is to invest basic research into mouse models of cancer," he argues. Clinicians might also use such a mouse model to test new treatments, he adds.

Achieving a Burkitt's mouse would not have been possible without previous work done by other NIAID scientists, Morse notes, particularly Janet Hartley, Ph.D., and Torgny Frederickson, Ph.D., who have spent years examining lymphomas in mice. The success also owes much to collaborator George Bornkamm, M.D., and his team at the Institute for Clinical Molecular Biology and Tumor Genetics in Munich, Germany.

Burkitt's lymphoma was first identified in 1958 by Denis Parsons Burkitt, an English surgeon in Africa who noticed it among the children there. The disease accounts for over half of all childhood cancers in Africa, where it affects about two out of 100,000 children every year. Incidence in Western countries is much lower, but has been on the rise with the spread of AIDS, since the cancer also strikes adults with compromised immune systems.

NIAID is a component of the NIH. NIAID supports basic and applied research to prevent, diagnose, and treat infectious and immune-mediated illnesses, including HIV/AIDS and other sexually transmitted diseases, tuberculosis, malaria, autoimmune disorders, asthma and allergies.

Press releases, fact sheets and other NIAID-related materials are available on the NIAID Web site at <http://www.niaid.nih.gov>.

Reference:

S Janz, HC Morse III, *et al.* Burkitt's lymphoma in the mouse. *Journal of Experimental Medicine* 92 (8):1183-90 (2000).

The UC Center for Animal Alternatives presents:

The Mouse in Science:

Cancer Research

Mice have been used in cancer research since 1894. Initially, mice were used for same-species tumor transplantations and drug treatment studies. In 1921, inbred strains that were predisposed to getting tumors were started and disseminated among cancer researchers. Many more strains of mice were originated beginning in 1929 with the founding of the Jackson Laboratory in Bar Harbor, Maine, now the largest supplier of mice.

In 1962, the discovery of a mutant mouse with low immunity led to human tumor transplantations, a valuable breakthrough for cancer research. A further breakthrough in the late 1980s led to transgenic mice, those whose genes have been altered to produce a desired characteristic. Oncogenes, or genes that cause cancer, could then be studied in greater detail.

Life in the Laboratory.

Mice adapt well to laboratory housing and can be housed socially or individually. Significant numbers can be housed in relatively little space because of their small body size. They possess a surprising genetic similarity to humans. These features, combined with a rapid rate of reproduction, make mice the mammal of choice for fine-tuned genetic manipulation. Mice with many different special features have been bred or created, including some described here.

Inbred Strains.

The inbreeding of mice predisposed to developing cancer has led to a variety of specialized strains. In 1921, Leonell Strong established many inbred strains that frequently

and spontaneously developed cancer. Serving as a virtually unlimited source of many types of tumors, these inbred mice have made it possible to study the growth and general characteristics of tumors.

Nude Mice.

The nude mouse is a major breakthrough for cancer research because it allows human tumors to be studied in another animal. The nude mouse, a hairless mutant discovered in 1962, is immunodeficient, and thus does not reject tumor transplantations from other species. It lacks a thymus, which is essential for the production of T-cells, **lymphocytes** that are essential to the immune system. By transplanting an actual human tumor into a nude mouse, the tumor can be studied in a whole animal system.

Before discovery of the nude mouse, human tumors were grafted and grown in immune-privileged sites, such as the anterior chamber of the eye, the brain and the cheek pouch. These locations are inconvenient, and the tumors are eventually rejected. The recessive *nu* gene, which is responsible for the lack of a thymus in nude mice, has since been introduced into many types of inbred strains of mice with other immunodeficiencies.

SCID Mice.

In 1983, mice with severe combined immune deficiency (SCID) were discovered. SCID mice are even more immunodeficient than nude mice. Tumors from other species are easily transplanted into SCID mice and will grow without being rejected. For certain specific tumors, SCID mice show improved transplantability over nude mice. In addition, SCID mice are ideal for the growth of **hybridomas** *in vivo* to produce a continuous supply of antibody (Ab). Sometimes referred to as a reagent, Ab is necessary for a wide range of diagnostic, clinical and experimental procedures.

Transgenic Mice.

In the late 1980s the methodology for engineering transgenic mice made it possible to create mice to address specific questions and problems. Transgenic mice result from genetically altered embryos: a gene or combination of genes is microinjected into developing oocytes. The genetic alteration affects the germ plasm, and subsequently can be transmitted to progeny. Through selective breeding, it then is possible to maintain a strain of mice consisting of individuals with particular traits of interest.

A specific trait, such as a predisposition to develop a particular type of tumor, can be introduced into a mouse strain by injecting into the embryo an oncogene, a gene that causes cancer. Transgenic mice permit the study of cancer in specific tissues, including initial tumor development.

Uses.

The purpose of cancer research is to understand tumor initiation and growth. This information helps researchers develop treatments, and eventually cures, for cancer. Many aspects of cancer research use mice, including:

*** Production of Tumors.**

Different: Types of cancer are induced through inbreeding or transgenic techniques (e.g., breast cancer).

Specific: Specific tumors from humans are transplanted into nude and SCID mice.

*** Therapy Testing.**

Chemotherapy: Mice with tumors are treated with different compounds to see if their tumors regress. Cancer cells from humans are also cultured in vitro to screen possible useful compounds.

Radiotherapy: Mice with tumors are exposed to radiation to see whether their tumors regress.

Immunotherapy: The immune system in whole animals is stimulated to treat cancer.

1. Monoclonal Antibodies (MAbs): MAbs are used to target cancer cells selectively.
2. Interferons: A species-specific, natural body substance that is secreted to fight viral infections, mouse interferon has been used against mouse leukemia and has been shown to stop cell division. Interferon shows promise in treating hairy-cell leukemia, non-Hodgkin's lymphoma, and other cancers.
3. Interleukins (IL): Mostly focusing on IL-2, researchers have tested its ability in vivo to kill tumor cells in mice. IL-2 appears to increase T-cell immune properties along with a generalized immune effect against cancer.

Carcinogenicity Testing.

Mice and other animals are used to test the cancer-causing ability of substances. Although mice are still widely used for these tests, the number of whole animals used in carcinogenicity testing has diminished. Faster, short-term tests are now used to screen substances. One such test uses cells growing in vitro to measure the ability of a substance to change cellular DNA. If it causes changes in DNA, it is considered a mutagen, or potential carcinogen.

Alternatives in Cancer Research.

Although no alternatives could completely replace the use of mice in current cancer research, uses of mice have already been reduced and refined. *In vitro* systems such as cell and tissue culture are the primary alternatives. Cell culture is less expensive than the use of whole animals and is easier to manipulate. For example, cancer-causing effects of radiation have been studied in cell culture. If whole animals (*in vivo*) were used, they would have to be irradiated and observed throughout their lifetime for tumor development -- a more costly and time-consuming procedure than the use of cell cultures. Changes in the growth of cells are good indicators for revealing when normal cells become tumor-producing.

Some aspects of cancer research require whole animal studies cells cannot mimic the physiology of a live animal. Clinical studies involving humans are another alternative. *In vivo*, *in vitro*, and clinical studies all combined produce the most informative results.

Recent Breakthroughs.

Research with both human patients and animal models is in progress, seeking to devise improved methods of treating cancer. With the development of recombinant DNA technology and gene therapy, a functioning gene is inserted into the cells of a patient in order to correct a genetic defect or to introduce a new function into the cell. This technique was approved for clinical trials in the late 1980s. It may become possible to use retroviruses as vectors to deliver the particular desirable genes into defective cells.

Adenoviruses are a type of virus that attack human cells; recently it has been found that they "turn off" the cancer-suppressing p53 gene. In cancerous cells, the p53 gene is defective: it fails to suppress cancer. Researchers have genetically engineered an adenovirus that attacks the defective p53 gene and protects the normal p53 gene. When human tumors in mice were injected with this adenovirus, the tumors regressed and disappeared. These initial results suggest that the method might also be effective in humans.

Glossary.

antibody a protein formed in reaction to an antigen, which it then attacks and destroys.

hybridoma. the cell produced by the fusion of an antibody-producing cell and a tumor cell.

interleukins. specialized substances that help to produce cellular immunity.



Products

Cantuzumab Mertansine (huC242-DM1/SB-408075) for Colorectal, Pancreatic, and Non-Small-C ll Lung Cancer

The American Cancer Society estimates that there will be 135,400 new cases of colorectal cancer, and that 56,700 Americans will die from the disease this year. For the 25% of patients for whom surgery is not an option, the outlook is bleak. Furthermore, about one-third of those who do undergo surgery suffer relapses before eventually succumbing to the disease. With the prognosis so grim, the need for improved colorectal cancer therapy is critical.

[Overview](#)

[Cantuzumab Mertansine](#)

[huN901-DM1/BB-10901](#)

[huMy9-6-DM1](#)

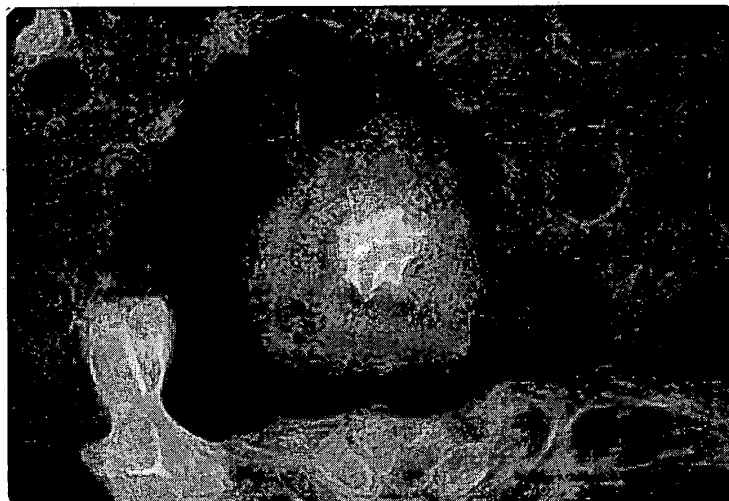
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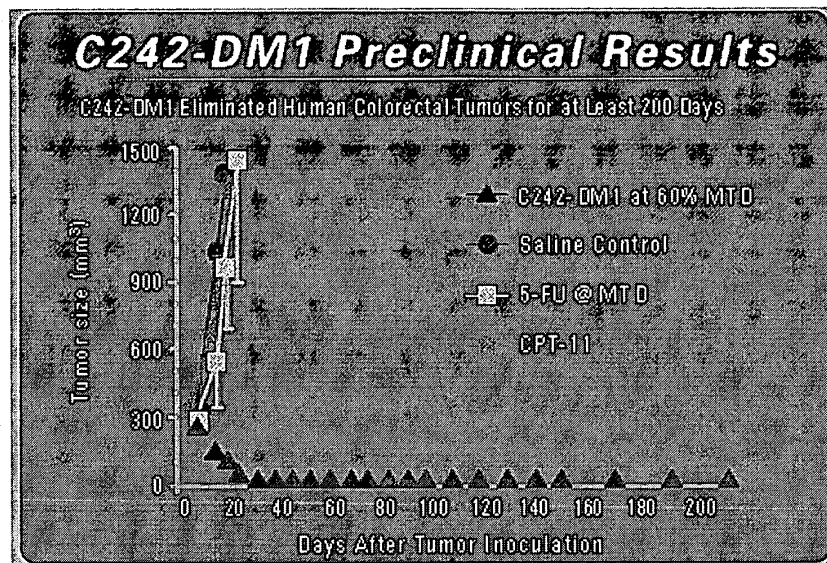
This image shows a colorectal cancer cell surrounded by healthy tissue. The cell is undergoing abnormal mitosis (or cell division) with multiple spindles, stained yellow-green, appearing in the nucleus.

A Breakthrough Treatment for Colorectal Cancer

ImmunoGen has developed a powerful treatment for colorectal cancer, utilizing DM1, a compound significantly more potent than current chemotherapeutic agents. ImmunoGen delivers DM1 directly to colorectal tumors by attaching it to a *monoclonal antibody*, huC242, that binds to a protein found on *colorectal cancer* cells. By linking huC242 to DM1, ImmunoGen has created a product that, in animal studies, is 100 times more potent than any other chemotherapeutic agent tested.

In published mouse studies, C242-DM1 completely eliminated transplanted human colorectal tumors in SCID mice at very low doses. Subsequent studies in non-human primates have shown the compound's safety and pharmacokinetic profile to be advantageous. These results are striking in light of the poor response of colon tumors to current chemotherapeutics of choice, including 5-FU and CPT-11 (see figure below), agents that have demonstrated only limited impact on the tumor. In additional preclinical studies, C242-DM1 has shown similar results in treating pancreatic and non-small cell lung cancer in SCID mice. The antibody component of this

product has been humanized to facilitate repeat dosing.



ImmunoGen's C242-DM1 colorectal TAP eliminated human colorectal cancer in SCID mice for at least 200 days.

In December 1999, ImmunoGen began a single-dose Phase I/II human clinical trial of this product in patients at the Cancer Therapy and Research Center in San Antonio under the direction of Anthony Tolcher, M.D. and Eric K. Rowinsky, M.D. We reported promising Phase I/II data in this first clinical trial of *cantuzumab mertansine* (huC242-DM1/SB-408075) at the American Society of Clinical Oncology (ASCO) meeting in May 2001.

This escalating-dose study was specifically designed to assess the safety and pharmacokinetic profile of the product. As this was the first time a TAP containing DM1, a substance 500- to 1000-fold more potent than existing chemotherapeutics, was injected into humans, it was administered on a once-every-three-week dosing schedule, a time period much longer than the expected half-life of the product, and started at very low doses. The study was conducted with 37, multiply-relapsed colorectal, pancreatic and non-small-cell lung cancer patients. All patients were heavily pre-treated with several rounds of chemotherapy and failed on average 3 recent regimens where their tumors continued to grow through treatment. Despite this, we saw minor responses in 2 patients (CAT scan-determined regressions of 33% of total tumor mass), persistent stable disease (i.e., no growth) of at least 18 weeks in 4 others and consistent decreases in colon cancer marker CEA in several more. The evidence of biological activity was confined to the higher dosage levels and only the later patients in the study received these higher dosage levels. Overall, the study results demonstrate a maximum tolerated dose of 235 mg/m² at which the TAP is well tolerated when given as a single bolus every three weeks. Further, no evidence of immunogenicity was observed.

In September 2000, the Company began a multi-dose study of this

product in a similar patient population at the University of Chicago Cancer Research Center. This study was designed to test safety and tolerability on a more frequent dosing schedule (once per week). Preliminary results of this study were presented at the AACR-NCI-EORTC Conference on Molecular Targets and Cancer in November 2001. The results showed favorable safety data from the initial 27 patients treated. Evidence of anti-tumor activity in several patients was also reported. Additional patients are being accrued.

Finally, in May 2001, we began enrollment for a third Phase I human clinical study of *cantuzumab mertansine*. This study is designed to evaluate *cantuzumab mertansine* when administered in a more dose-intensive regimen where patients are dosed three times weekly. The study is being conducted at the CTRC in San Antonio, Texas, under the direction of Anthony W. Tolcher, M.D. and Eric K. Rowinsky, M.D.

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Last Updated: December 21, 2001



Glossary

5-FU - 5-Fluorouracil.

Antibody - a protective protein produced in the body in response to exposure to foreign substances.

Antigen - a protein or carbohydrate capable of stimulating an immune response.

Apoptosis — also known as cell suicide; the natural process by which cells die in order to prevent the spread of disease. In disorders such as cancer, this cell death mechanism does not function properly, and cancer cells are allowed to proliferate.

Cancer — a group of more than 100 diseases caused by abnormal cells that grow and spread uncontrollably. Cancer can occur in any part of an animal where cells grow and divide. A mass, or collection, of cancer cells called a malignant tumor frequently grows rapidly, invading and destroying nearby tissues. Tumors may eventually metastasize, or spread to other parts of the body.

Chemotherapeutic — one of more than 50 drugs that are administered to stop the growth of or to kill cancer cells. Chemotherapeutic drugs are typically used to combat systemic cancers, those of the blood or lymph, or solid tumors that have spread to other parts of the body. More than a dozen cancers that formerly were fatal are now curable with chemotherapeutic drugs.

Cisplatin - a chemotherapeutic agent.

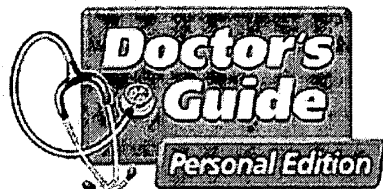
Colorectal cancer — cancer of the colon or rectum. Of the cancers that affect the large intestine, about 70 percent occur in the colon and about 30 percent in the rectum. These cancers are the second most common cancers overall. The American Cancer Society estimates that there were 131,600 new cases of colorectal cancer in the United States in 1998, and that 56,500 persons died of the disease.

CPT-11 - Irinotecan

Cytotoxic agent - agents that kill cells.

Etoposide - a chemotherapeutic agent.

huC242-DM1 — also known as SB-408075. ImmunoGen's lead TAP (tumor-activated prodrug). ImmunoGen delivers DM1 directly to colorectal, pancreatic, and non-small-cell lung cancer tumors by attaching it to an antibody, huC242, that binds to an antigen found on the cells. By linking



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Doctor's Guide

June 17, 1998

LA JOLLA, CA -- June 17, 1998 -- Results from two phase I studies and one preclinical study of Agouron Pharmaceuticals, Inc.'s oral anti-angiogenesis drug, AG3340 show the drug exhibits marked anti-tumour activity.

The data were reported today at the 10th European Organization for Research and Treatment of Cancer (EORTC) meeting in Amsterdam, The Netherlands.

In a phase I safety and tolerability study of AG3340 administered orally twice daily (BID) in patients with advanced cancer, including lung, prostate, kidney and colorectal cancers as well as sarcoma and melanoma, disease was stabilised in more than 25% of 47 evaluable patients who were treated for periods of 16 to 40 weeks.

Either two or three patients in each of five small dosing groups (5mg, 10mg, 25mg, 50mg and 100mg BID) comprising the 47 patients experienced stable disease. Three patients (one each with non-small cell lung cancer, renal carcinoma and melanoma) were found to have minor reductions in tumour volume. Nine other evaluable patients in two additional dosing groups (1mg and 2mg BID) had not yet received 16 weeks of treatment and are still under evaluation.

AG3340 was found to be generally well tolerated in this study. Expected musculoskeletal side effects, including arthralgias and body aches, occurred less frequently at doses below 25mg BID and were managed effectively by a rest from treatment followed by a dose reduction.

Based on the safety and tolerability data from this study, pivotal phase II/III clinical trials have been initiated using AG3340 in 5mg, 10mg, and 15mg doses given BID in combination with standard chemotherapy in patients with advanced non-small cell lung cancer or advanced hormone-refractory prostate cancer.

A separate phase I study found that AG3340 in combination with chemotherapy was generally well tolerated among patients with advanced prostate cancer who were resistant to hormonal therapies. This study evaluated the use of AG3340 -- in a dose of 25 mg BID -- in combination with Novantrone(R) (mitoxantrone) plus prednisone in 15 patients with advanced prostate cancer. In this ongoing study, nine patients have received the combination treatment for more than ten weeks; seven patients have received treatment for more than 18 weeks.

Pharmacokinetic analysis of the available data confirmed that AG3340 blood levels are not affected by administration with mitoxantrone. No unexpected side effects occurred in the patients in this study.

In a separate preclinical study, AG3340 was found to be a potent inhibitor of the growth of chemotherapy-resistant human non-small cell lung cancer tumours in mice. Here, administration of AG3340 resulted in a dose-dependent decrease in tumour growth by up to 65% as compared to controls. Paraplatin(R) (carboplatin), a currently available chemotherapeutic agent, demonstrated a similar amount of anti-tumour effect at toxic doses, whereas AG3340 inhibited growth at well-tolerated doses.

The study also demonstrated that the combination of carboplatin and AG3340 was more effective than either agent alone, suggesting that the combination of the two drugs could provide beneficial clinical results.

A key action of AG3340 was also demonstrated by finding a 77% reduction in the formation of new tumour-associated blood vessels. Neovascularization, or new blood vessel formation (angiogenesis), is required to support growing tumours.

AG3340 is an orally active, synthetic molecule designed to inhibit the growth, invasion and metastasis of solid tumours by inactivating certain members of a family of enzymes known as matrix metalloproteases (MMPs). AG3340 selectively inhibits those MMPs believed to be involved in tumour progression. A primary goal of the clinical studies of AG3340 is to determine whether this distinctive selectivity results in a favourable clinical profile of safety and efficacy.

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April 30, 1999

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UNMC Home

Breakthrough on Pancreatic Cancer

A UNMC researcher has gained national attention with his findings on a new drug used to treat pancreatic cancer.

Changnian Liu, M.D., Ph.D., assistant professor in the Department of Cell Biology and Anatomy, recently presented his data at the American Association for Cancer Research 90th Annual Meeting in Philadelphia.

Since then, more than 20 newspapers, magazines and Internet services have reported on Dr. Liu's findings.

Dr. Liu and his colleague, Shantaram Joshi, Ph.D., associate professor of cell biology, along with collaborators at Rush-Presbyterian-St. Luke's Medical Center in Chicago, have developed Virulizin, an immunotherapeutic agent for pancreatic cancer.

They believe Virulizin is as effective as the standard treatment for pancreatic cancer, but has a much better safety profile.

"Our results show that the activity of Virulizin against pancreatic cancer is comparable to that of the current best chemotherapeutic drug (Gemcitabine) and that it is well tolerated by patients," Dr. Liu said.

Pancreatic cancer traditionally was treated with a standard chemotherapy drug called 5-FU, but with poor results. With the introduction of another drug called Gemzar, treatment improved and survival rates doubled to six months.

Pancreatic cancer is considered one of the most aggressive types of cancers and accounts for 28,000 American deaths each year. Only 20 percent of cases are detected in time to attempt surgical removal of the tumor. Less than 10 percent of patients survive for one year after diagnosis. The median survival rate for patients who can not have tumors removed

surgically is three to four months.

Dr. Liu, whose research is supported by Lorus Therapeutics Inc., a Canadian biopharmaceutical company in Toronto, has developed an immune-system booster that is derived from cow bile. Although it is not a cure for most patients, the drug seems to work as well as standard medicine with fewer side effects. The introduction of Virulizin improves survival rates to a little more than six months.

In a preclinical study in mouse models of human pancreatic cancer at UNMC, Dr. Liu and his colleagues found that Virulizin significantly inhibited tumor growth. Tumors among treated mice were 60 percent the size of the tumors in untreated mice. When the chemotherapy agent Gemcitabine was added to the treatment, tumors in the treated mice were only 26 percent of the size of the untreated mice.

Phase I/II clinical trials were conducted at Rush-Presbyterian-St. Luke's Medical Center. Twenty-six patients who had failed standard therapies were treated. Seven of the 26 patients achieved a stable disease, which means their tumor did not grow. One patient achieved a complete response, meaning his or her tumor disappeared for a minimum of four weeks and is still alive 22 months later.

Virulizin works by boosting the power of macrophages, scavenger cells that fight cancer directly and also rev up other parts of the immune system.

"This is the first report that an immunotherapeutic agent has produced improved quality of life and overall survival in patients with advanced pancreatic cancer," Dr. Liu said. "Additional studies are needed to evaluate the effectiveness of Virulizin treatment in all patients with pancreatic cancer and not just those with advanced disease."